RESEARCH PAPER

Enhanced QuEChERS-LC-MS/MS Protocol for Precise Quantification of Clorazepate, Diazepam, and Etizolam in Urine Samples

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Abstract:
In recent years, India has seen multiple incidents of anxiolytic toxicity. Because drug misuse is inextricably related to other major crimes such as organised crime, suicide, murder, and rape, it is now acknowledged as a complicated issue. Benzodiazepines, such as Clorazepate and Diazepam, are among the most commonly reported cases of anxiolytic drug misuse in forensic laboratories.
In this study, LC-MS/MS was combined with QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe). The dSPE analytical approach detects clorazepate, etizolam and diazepam at six different concentrations in urine. Anxiolytics were discovered and measured using the ESI+ mode. The procedure was verified with limits of detection (LOD) of 9.42-10.40 ng/ml and quantification (LOQ) of 28.15-31.23 ng/ml for each analyte. The linear calibration curve from 5 to 200 ng/ml was achieved with an R^2 value greater than 0.998. Furthermore, the approach was very accurate and robust, with a recovery range of 70-120% based on SWGTOX standards. This study successfully reported the extraction of benzodiazepines for the first time with higher recovery rate of (>75%) in urine.This work offers a reliable and effective method to extract and identify antianxiety drugs from urine.

Keywords: anxiolytic drugs, poisoning, QuEChERS extraction technique, biological matrices, LC-MS/MS
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1. BACKGROUND:

It is very important for forensic investigations, therapeutic drug monitoring, and clinical toxicology to have an exact identification and quantification of benzodiazepines in biological samples. Clorazepate, etizolam and diazepam are among the common benzodiazepines that are prescribed due to their sedative, anxiolytic and anticonvulsant properties. However, owing to their potential for abuse and involvement in drug facilitated crimes there is a need for reliable analytical methods that could identify these drugs when they are present in biological matrices particularly urine. [1, 2]. National Survey conducted in 2022 on Extent and Pattern of Substance Use showed that about 1.21% of adults (eleven million people) and another 0.58% of adolescents (almost two hundred thousand individuals) used benzodiazepines to treat anxiety. [3].
Benzodiazepines, commonly referred to as "benzos," constitute a class of psychoactive compounds that have played a pivotal role in the field of medicine and pharmacology since their introduction in the mid-20th century. [4]. However, beyond their therapeutic benefits, benzodiazepines are not without concerns, particularly concerning their potential for toxicity. This paper focuses on exploring the toxicological aspects of benzodiazepines, by extracting the drug from human urine using QUEChERS extraction technique followed by LC-MS/MS.

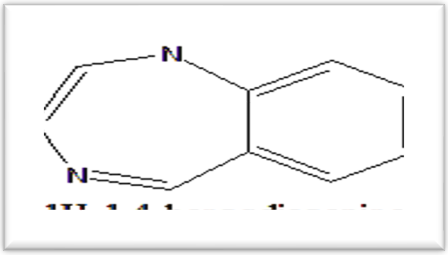

Fig 1: Structure of Benzodiazepines:

Benzodiazepines exert their therapeutic effects by enhancing the inhibitory actions of gamma-aminobutyric acid (GABA) in the central nervous system, ultimately promoting relaxation and sedation.

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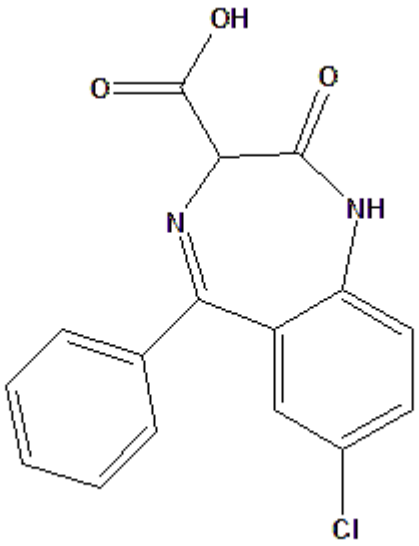
While these medicines have played a key role in psychiatric and medical treatment, they also carry the risk of misuse, dependence, and adverse effects that can escalate to toxicity. Understanding the toxicological properties of benzodiazepines is crucial for healthcare providers, clinicians, and policymakers to ensure the safe and responsible use of these drugs. [5]. For this study, three benzodiazepine drugs such as Clorazepate, Etizolam and Diazepam were used as they are widely prescribed by doctors in India.

Clorazepate, also known by its brand name Tranxene, is a benzodiazepine medication used primarily for the treatment of anxiety disorders. Its chemical structure is characteristic of benzodiazepines, a class of psychoactive compounds known for their anxiolytic, sedative, and muscle-relaxant properties. [6]. The chemical structure of clorazepate can be described as follows:

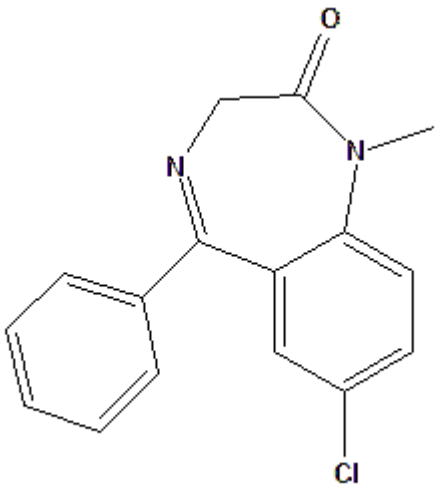


7-chloro-2-oxo-5-phenyl-1,3-dihydro-1,4-benzodiazepine-3-carboxylic acid

Fig 2: Clorazepate Chemical Structure

Clorazepate has a fused-ring structure that consists of a diazepine ring and a benzene ring. The diazepine ring, also known as a seven-membered ring, is a defining feature of benzodiazepines. The chemical formula for clorazepate is C16H11ClN2O4, and it has a molar mass of approximately 314.72 g/mol. The specific chemical structure of clorazepate imparts its pharmacological properties, including its ability to enhance the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) in the central nervous system, leading to its anxiolytic and sedative effects. [7].

Diazepam, a widely used benzodiazepine medication with brand names such as Valium, is known for its anxiolytic, sedative, and muscle relaxant properties. Its chemical structure is characteristic of benzodiazepines, a class of psychoactive compounds. [8]. The chemical structure of diazepam can be described as follows:

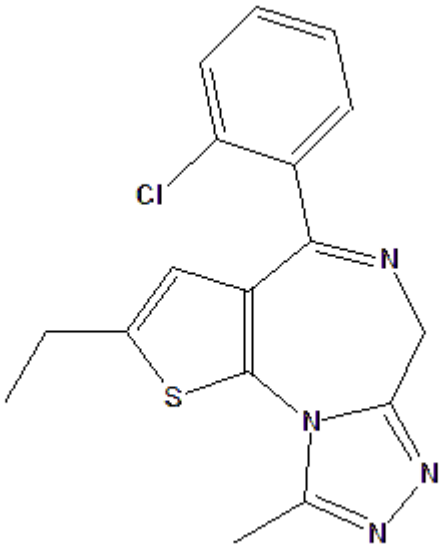


7-chloro-1-methyl-5-phenyl-3H-1,4-benzodiazepin-2-one

Fig 3: Diazepam Chemical Structure:

Diazepam features a fused-ring structure comprising a diazepine ring and two benzene rings. The diazepine ring, a seven-membered ring, is a hallmark of benzodiazepines. The chemical formula for diazepam is C16H13ClN2O, and it has a molar mass of approximately 284.74 g/mol. The unique chemical structure of diazepam enables it to enhance the activity of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) in the central nervous system, which underlies its anxiolytic and sedative effects. [9].

Etizolam is a thienodiazepine derivative and a thienodiazepine analogue. It is a psychoactive medication with anxiolytic and sedative properties, often used in the management of anxiety and sleep disorders. [10]. The chemical structure of etizolam can be described as follows:



4-(2-Chlorophenyl)-2-ethyl-9-methyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepine

Fig 4: Etizolam Chemical Structure:

Etizolam features a thienodiazepine core structure, which is a seven-membered diazepine ring fused with a thiophene ring. This thienodiazepine ring is characteristic of etizolam and differentiates it from traditional benzodiazepines. The chemical formula for etizolam is C17H15ClN4S, and it has a molar mass of approximately 342.85 g/mol. Etizolam's structural similarity to traditional benzodiazepines enables it to act on gamma-aminobutyric acid (GABA) receptors in the central nervous system, resulting in its anxiolytic and sedative effects. [11].

Benzodiazepines toxicity presents in various forms from overdose to drug-drug interactions and withdrawal symptoms. Overdosing with co-ingestion of other substances, can lead to severe central nervous system depression, respiratory depression, and, in extreme cases, death. [12]. Furthermore, benzodiazepines can interact with other medications and substances, potentially amplifying their toxic effects. [13]. Therefore, this has become the topic of interest for forensic toxicologist to develop new and modified method for detection of this drug from biological samples.

QuEChERS has increasingly been used in toxicological field since the technique offers high sensitivity, specificity and structural information about analytes in complex biological samples. Better results can be obtained in LC-MS/MS analysis when sample preparation techniques are made more robust. One of such methods that has become very popular is the QuEChERS extraction process. This was first designed for use in pesticide residue analysis of agricultural products, but today it is widely used for different purposes including the recovery of drug compounds from biological samples. [14].

Jessica et al., develop an efficient, reliable and cost-effective method for the extraction of benzodiazepines from biological samples using the QuEChERS technique. The focus was on optimizing this method for use in forensic toxicology to improve the detection of benzodiazepines in complex biological matrices. The method provided low limits of detection (LOD) and quantification (LOQ), which are essential for forensic applications where trace amounts of drugs need to be identified. [15]. The work of Jacqueline et al., describes the development and optimization of a QuEChERS extraction method for the detection and quantification of 20 antidepressants in postmortem blood samples using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The optimized protocol included the use of acetonitrile as extraction solvent and a combination of buffer salts (magnesium sulfate and sodium acetate) to improve phase separation. The method demonstrated high sensitivity, specificity, accuracy and precision,

making it a valuable tool for forensic toxicology. The application to real forensic cases underlined its practicability and effectiveness and provided reliable data for the determination of antidepressants in post-mortem examinations. [16]. Luca's et al., focuses on the adaptation and optimization of the QuEChERS extraction method for the analysis of drugs and benzodiazepines in blood samples using ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). The study aims to overcome the challenges associated with the complex nature of blood matrices and to improve the reliability and efficiency of drug analysis in forensic toxicology. Acetonitrile was selected as the primary extraction solvent as it enables effective protein precipitation. [17]. Matsuta et al., aim was to simplify the traditional QuEChERS method by integrating the extraction and purification steps into a single stew process. This approach reduces the number of steps and thus minimizes sample loss and the risk of contamination. [18]. Uddin et al., optimized the SPE-HPLC method for the efficient extraction of benzodiazepines and their metabolites from biological matrices. They developed and optimized this to achieve high resolution and sensitivity for the separation and quantification of the analytes. The validated method showed excellent performance in terms of sensitivity, specificity, accuracy and precision for all matrices tested. The LOD and LOQ were sufficiently low to detect and quantify traces of benzodiazepines and their metabolites in biological samples. [19].

This research paper aims to provide a comprehensive examination of the toxicological dimensions of benzodiazepines from human biological sample, incorporating new and enhanced QuEChERS extraction technique with LC-MS/MS. By delving into these aspects, this paper seeks to offer insights that can guide forensic laboratories/toxicologist in their efforts to quickly and robustly detect BZDs drugs from urine samples.

The purpose of this study is to develop and validate an extraction technique based on QuEChERS which can be used for the simultaneous determination of clorazepate, etizolam and diazepam in urine samples utilizing LC-MS/MS.

2. Materials and Methods:

2.1 Chemical and Reagents:

Every chemicals and reagents used in this study were of analytical grade. For HPLC, methanol and formic acid were bought from Sigma Aldrich, St. Louis, MO, USA for dispersive solid-phase extraction (d-SPE): EN QuEChERS salt pouch (Agilent: Santa Clara, CA, USA) was supplemented with sodium citrate tribasic dihydrate (1 g), sodium citrate dibasic sesquihydrate (0.5 g). Anhydrous sodium acetate., anhydrous magnesium sulphate., sodium chloride., C18 column., sodium acetate., and primary secondary amine (PSA) were also procured from Sigma Aldrich, St. Louis, MO, USA. Standards of clorazepate, diazepam and etizolam were purchased from SIMA LABS (Sophisticated Industrial Materials Analytic Labs Pvt. Ltd.), New Delhi, India

2.2 Apparatus and Equipment:

Equipment includes a vortex, analytical balance, tabletop centrifuge, centrifuge tubes, sonicator, adjustable pipettes, and 10 mL volumetric flasks. For LCMSMS: Agilent 6470 B; Analytical column: Poroshell 120 with bonded phase EC-C18 (2.7 µm, 3mm x 150 mm). The capillary column and mobile phase filtration assembly were employed in the investigation.

2.3 Methodology:

2.3.1 Mobile Phase Preparation: 5mM Ammonium formate + 0.1% formic acid in water was prepared by transferring 1 ml of formic acid to a 1000 ml volumetric flask, to which, 315.3 mg Ammonium formate was added and diluted to 1000 ml with water. Sonicate for around 5 minutes to disintegrate and it remains stable for 5 days. Another solvent was 100% methanol.

2.3.2 Stock standard of Drugs residues preparation: In a 10 ml volumetric flask, weigh out the equivalent of 10 mg of drug standard. For different drug standards different volumetric flasks were used. Then, dissolve it in HPLC-grade acetonitrile to make the volume up to 10mL. Label contained the standard's name, concentration, preparation and expiration dates. Marked the expiration date and keep the solution at -20°C in a deep freezer.

2.3.3 Preparation of Drugs Intermediate Standard (IS) Mixture Solution: Pipette out 100µl of drugs from each stock solutions into 10mL volumetric flask. The total volume added were 300µl. Fill the flask with acetonitrile to make up the volume 10mL. The flask was swirled several times to make a homogeneous intermediate solution of drugs. The final volume of each drug in the solution was 10µg/mL.

2.3.4 Preparation of working standard dilution for calibration curve: Working standard of 10.0 mg/L, 1.0 mg/L and 0.1 mg/L was prepared with acetonitrile. The working standard ranged from 5 to 200 ng/mL to construct the calibration curve standard spiked in the various concentrations. Working solutions of the mix standards were prepared by serially diluting the stock solution at six different concentration levels ranging from 5, 10, 20, 50, 100 to 200 ng/mL for all drugs for calibration.

2.3.5 Sample Collection: The samples for this study were collected in July 2024. Known volume of urine samples (10mL) collected from healthy individuals in clean, sterile containers. Midstream urine was preferred to avoid any contamination, and samples were labelled immediately with date and time. Prior approval was taken from ethical committee to carry research on human biological samples. The samples were spiked with

known concentration of working standard solution of drugs.

2.4. Extraction Procedure:

The optimisation of the QuEChERS extraction process includes two approaches:

First, partition by adding 10mL Milli Q water to a 50mL centrifuge tube containing 5mL of homogenised urine sample spiked with clorazepate, diazepam, and etizolam at doses of 5, 10, 20, 50, 100, and 200 ng/mL. This mixture was diluted with 10mL of methanol. To homogenise the sample, the mixture is shaken for 10 minutes using a wrist motion shaker. The EN QuEChERS salt pouch [sodium citrate (1 g), 4 g MgSO4, 1 g NaCl, sodium citrate dibasic sesquihydrate (0.5 g)] is added to the homogenised liquid in a 15 mL centrifuge tube and vortexed for 1 minute. The sample was centrifuged at 6000 rpm for 10 minutes at 2-8°C using a refrigerator-based centrifuge. 6 mL of the supernatant layer was removed from the partitioning and placed in a fresh 15 mL centrifuge tube.

Second, cleanup is accomplished via dispersive SPE: 500 mg of magnesium sulphate is used to clear the supernatant layer, eliminating water molecules and moisture from the sample. 250 mg of primary and secondary amine (PSA) is then added to simplify the compound. The material was then centrifuged at 6000 rpm for 6 minutes at 2–8 °C after being vortexed for 2 minutes. Once more, the layer of supernatant was moved into a tube with 150 mg of MgSO4, vortexed, and centrifuged for five minutes at 12,000 rpm. For every clean-up, a 200 uL aliquot of the finished extract was transferred to a separate vial, and 10 uL of each sample was injected straight into the LCMS/MS device.

2.5 LC-MS/MS Conditions:

Experimental analysis of this research is performed on Agilent 6470B, LC-MS/MS (Agilent Technologies Inc., Santa Clara, CA, USA), in positive-ion mode. Parameters used for this analysis were as follows: precursor ion scans (100-500 m/z); 0.3–2.0 V ramping collision energy (Smart Frag); 4 amu isolation width; one MS and one MS/MS spectra. Sample solution injection volume: 10 uL Flow rate: 0.4 mL/min Thermal stability was assessed for 30 minutes at 220°C using temperature increase mode. Poroshell 120 with bonded phase EC-C18 (2.7 µm, 3 mm x 150 mm) was the analytical column utilised, and the temperature was adjusted to 40°C. The MRM (multiple reaction monitoring) modes, which had their transitions and collision energies adjusted, were used to produce the MS/MS data in order to assess and quantify clorazepate, diazepam, and etizolam.

3.RESULTS AND DISCUSSION:

3.1. Optimisation of QuEChERS method.

Although the QuEChERS technique is exploratory and descriptive, its efficacy is dependent on a limited number of criteria. Because they provide flexibility in introducing changes to conventional methods and improving the extraction process, studies such as these are essential for evaluating specific parameters, including the extraction solvent to be used, sample/solvent ratio, pH, type of agitation, type and quantity of partition salts, and cleaning sorbents. [20]

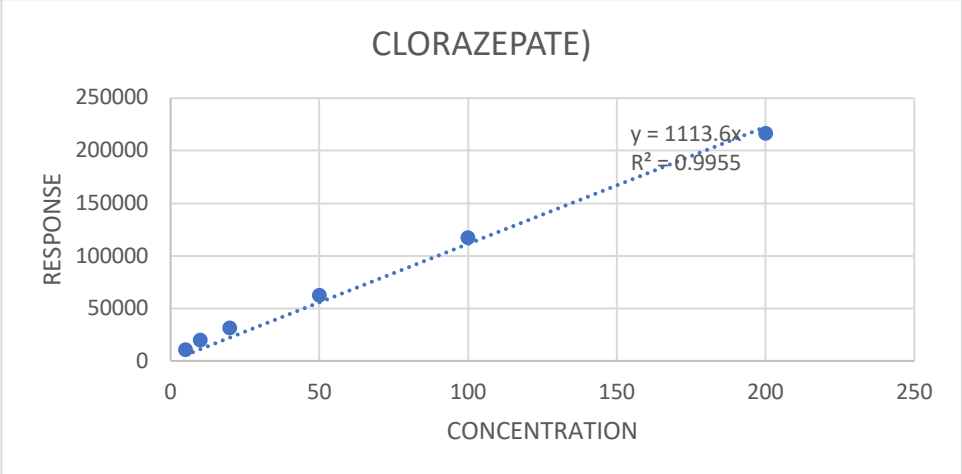


Figure 5: Calibration curve of Clorazepate in Urine

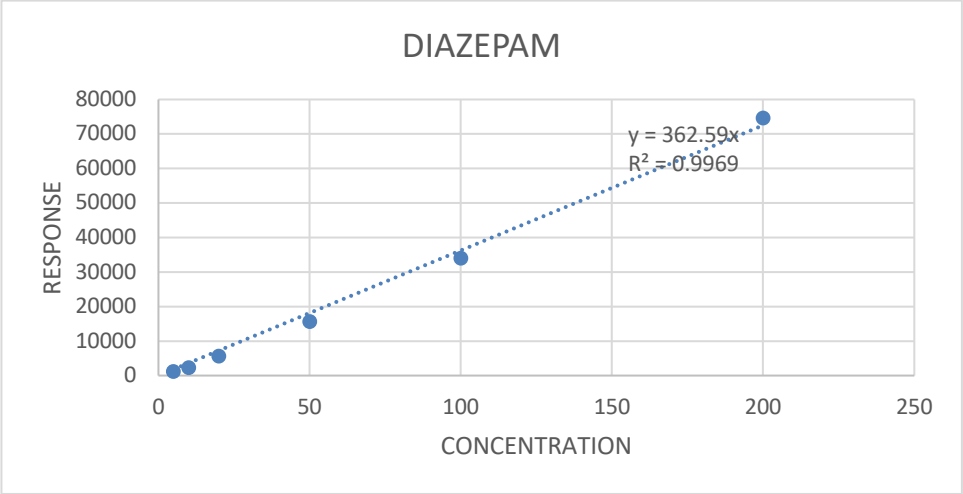


Figure 6: Calibration curve of Diazepam in Urine

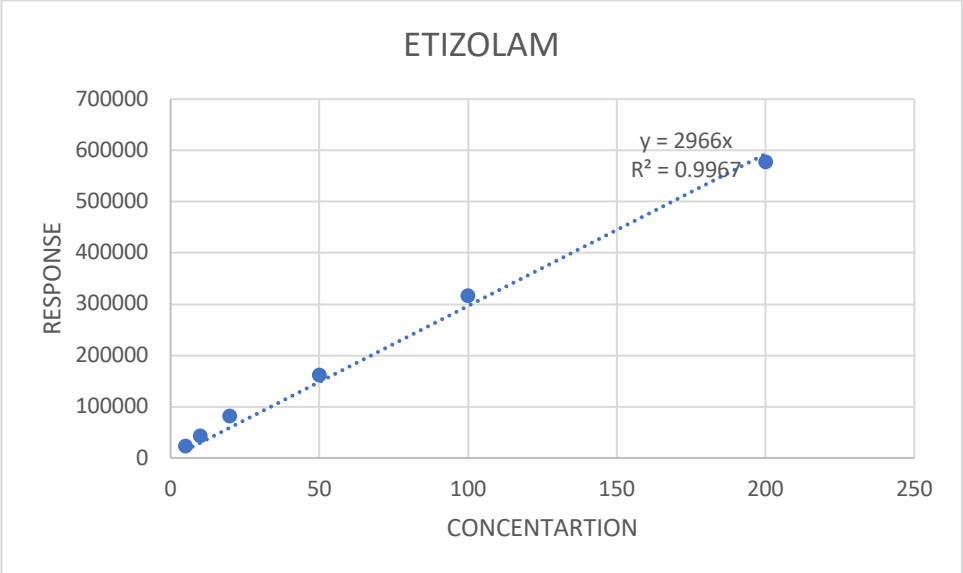


Figure 7: Calibration curve of Etizolam in Urine

A number of factors were thoroughly examined in order to optimise analyte recovery and reduce matrix effects while using the QuEChERS extraction technique for the detection of anxiolytic medications in urine.

A comparison of the statistical significance and amplitude of characteristics associated with the effects on the response (chromatographic peak areas) derived from the normal plots for each anxiolytic drugs served as the basis for the initial assessment. Typical calibration plots for the urine samples of clorazepate, diazepam, and etizolam are shown in Figures 5, 6, and 7. The linear graphs with the goodness of fit parameter (R^2) in the range of 0 to 1 show that the regression's predictions accurately predicted the data.

Ideal pH Level for Extraction: A pH range of 4 to 8 was used to determine the extraction effectiveness. The greatest recovery rates were seen at pH values between 5 and 6. Analytes exhibited great stability at this pH without becoming too ionised, which made it easier for them to partition into the organic phase during extraction. [21, 22].

Types of Salt and Their Impacts: Salts are frequently used in QuEChERS extraction procedures to enhance analyte partitioning and promote phase separation, both of which are essential for removing medications from the urine matrix.[23]. Salts Examined: Magnesium sulphate (MgSO4), sodium chloride (NaCl), and a mixture of sodium citrate and disodium hydrogen citrate were among the several salt mixes that were tried. In order to effectively remove water from the organic layer, improve phase separation, and concentrate the analytes, $MgSO_4$ was necessary. The "salting out" effect of NaCl, which decreases solubility in the aqueous phase and encourages medication partitioning into the organic layer, was examined. The best extraction efficiency was achieved by combining $MgSO_4$ and NaCl, which improved phase separation and reduced the possibility of interference from polar urine components. [24].

Effective extraction, phase separation, and stability across a variety of anxiolytic medications are supported by the final protocol's 1g $MgSO_4$ to 2g NaCl ratio +100mg trisodium citrate dihydrate + 100mg disodium

hydrogencitrate sesquihydrate and 2:1 (methanol: Urine)- Optimal Solvent Volume. [25].

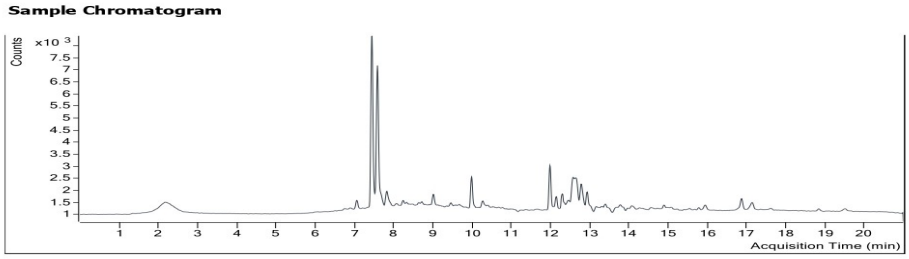
Ideal choice of sorbent: For the final technique, a combination of PSA and C18 sorbents was chosen because it provided the perfect balance, successfully lowering matrix effects without sacrificing target analyte recovery.

In the LC-MS/MS study, the PSA/C18 combination greatly reduced ion suppression or enhancement by selectively eliminating the majority of interferences.[26]. Methanol was used instead of acetonitrile because it offers mild protein precipitation and is miscible with water.

3.2 Optimization of LC-MS/MS Conditions:

The clear and reproducible baseline separation of the targeted anxiolytic medications from urine matrix components were obtained from the LC-MS/MS analysis. For each anxiolytic medicine, the optimized technique yielded distinct, sharp peaks that were easily distinguished from other urine constituents. The matrix effects were significantly lessened by the QuEChERS extraction technique in conjunction with a PSA/C18 clean-up phase, which eliminated polar and non-polar interferences that support ion suppression.

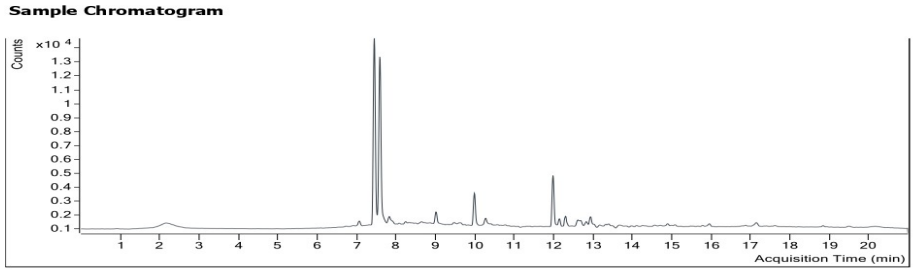
A variety of collision energy voltages was used to identify each sample's two distinct product ions after each precursor ion was seen. The quantifier ion was the peak with the second-highest intensity, and the qualifier ion was one of the transitions. Then, multiple reactions monitoring (MRM) transitions and dwell periods were used to automatically adjust the settings.



Quantitation Results

Compound	RT	Response	Conc	Unit
Clorazepate	7.452	10313	3.2748	mcg/l
Etizolam	7.591	23169	3.7315	mcg/l
Diazepam	9.020	1187	6.1755	mcg/l

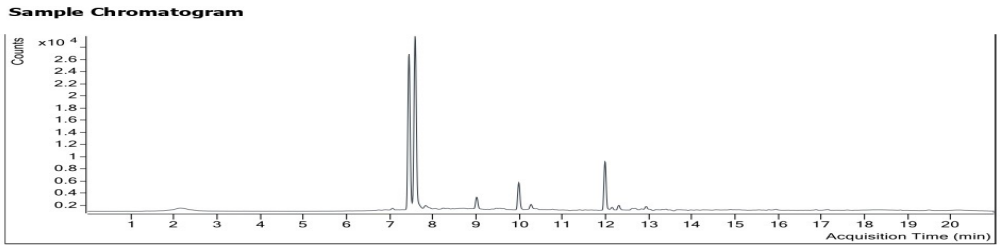
Figure 8: LC-MS/MS chromatograms (acquisition time vs. counts) of CZP, EZM, and DZP at a concentration of 5 ppb.



Quantitation Results

Compound	RT	Response	Conc	Unit
Clorazepate	7.452	19831	12.0593	mcg/l
Etizolam	7.600	43262	10.5837	mcg/l
Diazepam	9.020	2288	9.2143	mcg/l

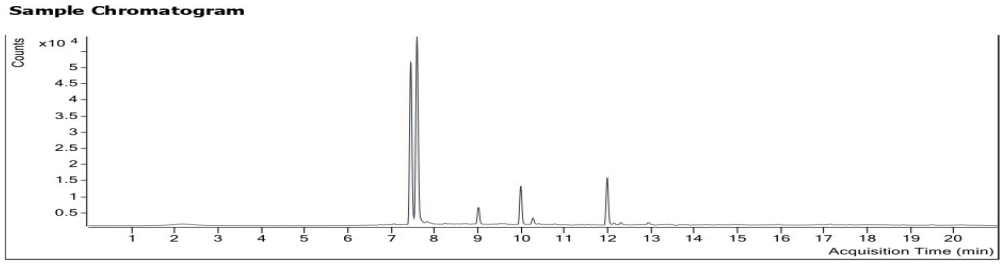
Figure 9: LC-MS/MS chromatograms (acquisition time vs. counts) of CZP, EZM, and DZP at a concentration of 10 ppb



Quantitation Results

Compound	RT	Response	Conc	Unit
Clorazepate	7.452	31002	22.3701	mcg/l
Etizolam	7.591	81361	23.5761	mcg/l
Diazepam	9.020	5582	18.3127	mcg/l

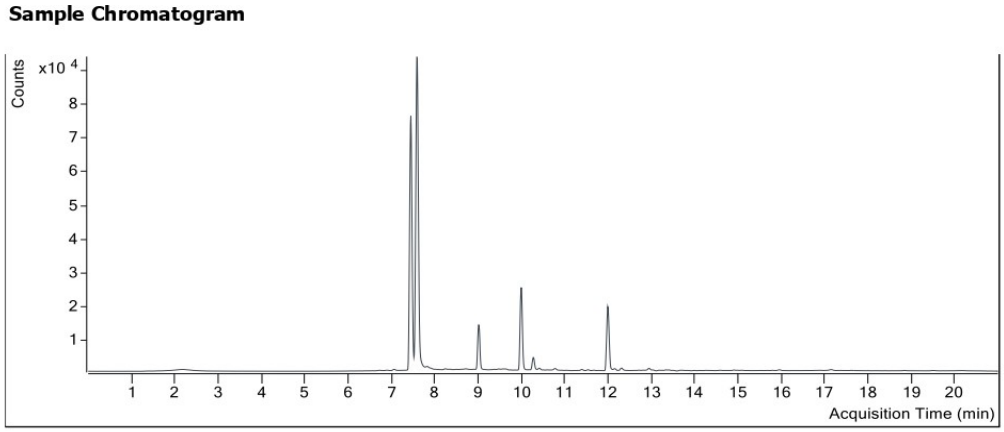
Figure 10: LC-MS/MS chromatograms (acquisition time vs. counts) of CZP, EZM, and DZP at a concentration of 20 ppb.



Quantitation Results

Compound	RT	Response	Conc	Unit
Clorazepate	7.452	62638	51.5703	mcg/l
Etizolam	7.600	161376	50.8621	mcg/l
Diazepam	9.020	15580	45.9223	mcg/l

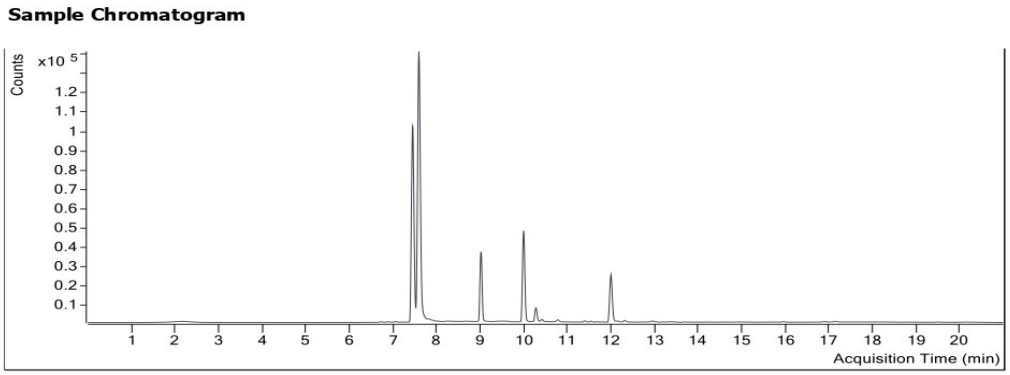
Figure 11: LC-MS/MS chromatograms (acquisition time vs. counts) of CZP, EZM, and DZP at a concentration of 50 ppb.



Quantitation Results

Compound	RT	Response	Conc	Unit
Clorazepate	7.452	117400	102.1148	mcg/l
Etizolam	7.600	316051	103.6083	mcg/l
Diazepam	9.020	33895	96.5015	mcg/l

Figure 12: LC-MS/MS chromatograms (acquisition time vs. counts) of CZP, EZM, and DZP at a concentration of 100 ppb.



Quantitation Results

Compound	RT	Response	Conc	Unit
Clorazepate	7.461	216529	193.6107	mcg/l
Etizolam	7.600	577126	192.6382	mcg/l
Diazepam	9.028	74586	208.8738	mcg/l

Figure 13: LC-MS/MS chromatograms (acquisition time vs. counts) of CZP, EZM, and DZP at a concentration of 200 ppb.

Besides optimizing the MS settings, the composition of the mobile phase, specifically the concentration and pH of the buffer solution—is essential for enhancing quantification specificity. These factors influence the shape of chromatographic peaks and improve the ionization efficiency of analytes in MS analysis. Compared to water, using 5 mM ammonium acetate resulted in narrower, more symmetrical peaks. Additionally, the addition of 0.1% formic acid notably increased the signal responses of clorazepate, etizolam, and diazepam.

Methanol proved more effective than acetonitrile for elution. After extensive testing, a mobile phase consisting of methanol, 5 mM ammonium acetate, and formic acid in a 35:65:0.1 ratio (v/v/v) was selected for consistent sensitivity and optimal retention of clorazepate, etizolam, and diazepam. Under ideal LC conditions, the retention times for these drugs in urine samples were established. MS parameters were optimized using a 100.0 ng/mL tuning solution in both positive and negative ionization modes. Positive ionization mode provided greater sensitivity with minimal background noise compared to the negative mode. The detected precursor/product ion transitions for clorazepate, diazepam, and etizolam were 140–271 m/z, 154–285 m/z, and 205.2–342.8 m/z, respectively. The MS optimization results are illustrated in Figures 14, 15, and 16.

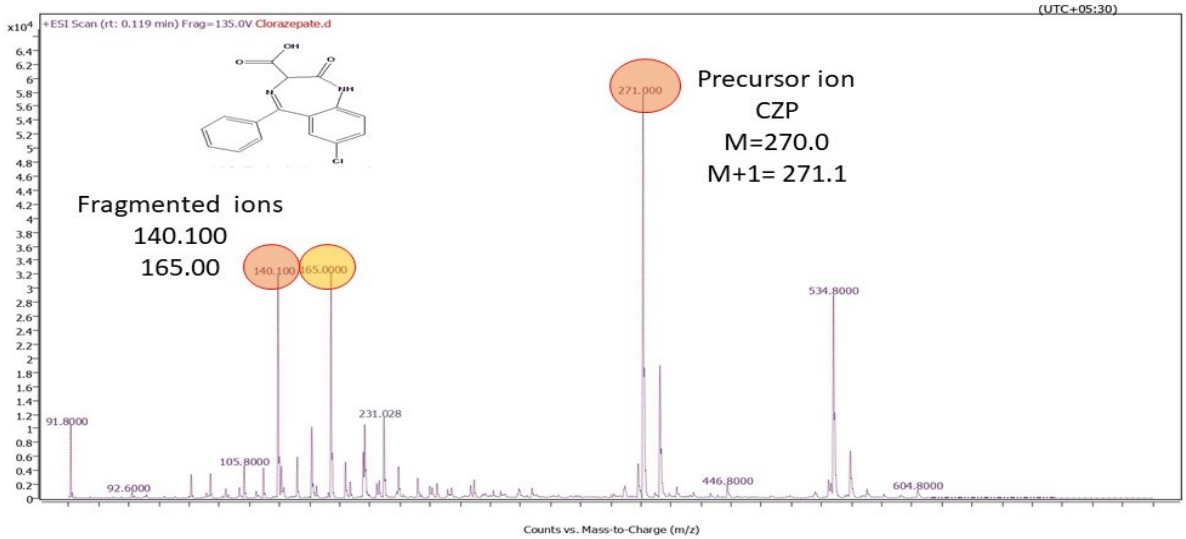


Figure 14: Optimisation of MS/MS parameter for Clorazepate

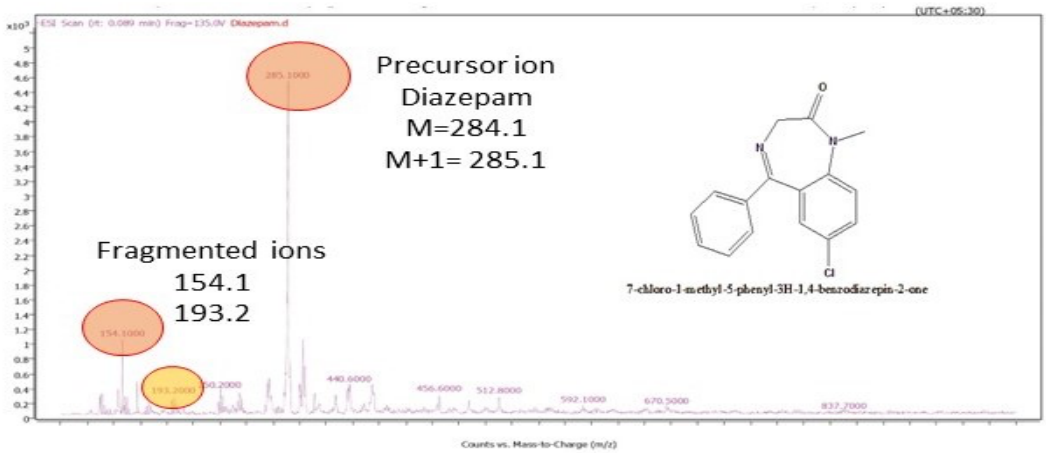


Figure 15: Optimisation of MS/MS parameter for Diazepam.

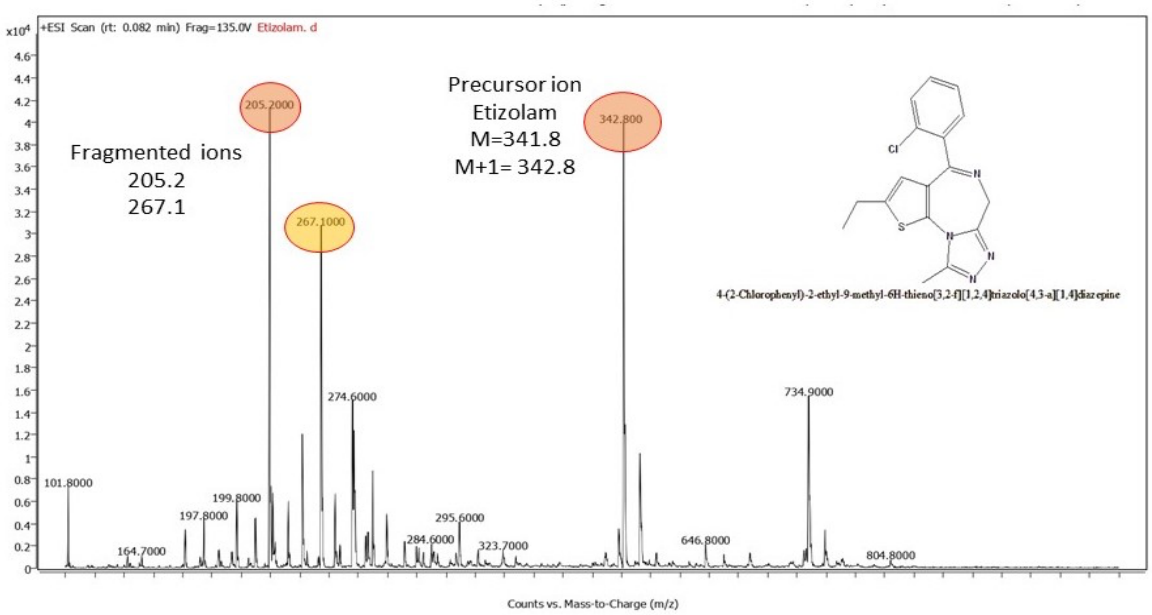


Figure 16: Optimisation of MS/MS parameter for Etizolam.

3.3 Method Validation:

To evaluate the process, an analysis was performed on the blank urine sample at six different doses. No interference peaks were seen to us. Time of retention for clorazepate, etizolam and diazepam were 7.45, 7.60 and 9.03, respectively. It took eight minutes for the sample to run.

3.3.1 Assessment of LOD and LOQ and Calibration method:

The accuracy of the method's linearity was demonstrated by creating a calibration curve by combining the peak area-to-standard internal ratio of analytes at six different concentrations.

The statistical software GraphPad Prism V9.0 was used to generate calibration curves from peak-area to standard internal ratios of analytes at six concentration levels. This was done to illustrate the linearity of the suggested method. The concentration range included the therapeutic concentration for all drugs, and the study's working range was 10 ng/mL. A linear regression coefficient R2>0.9999 indicates a strong model adjustment fit.

DRUGS	BIOLOGICAL MATRIX	R^2	SLOPE	INTERCEPT	RSD %	PRECURSSOR ION	PRODUCT ION	RETENTION TIME
Clorazepate	Urine	0.9993	0.9747	1.6224	0.844	271.0	140.1, 160.0	7.4
Diazepam	Urine	0.9992	0.97761	1.4374	1.833	285.1	154.1, 193.2	9.0
Etizolam	Urine	0.9991	1.0326	2.09	6.278	342.8	205.2, 267.1	7.6

Table 1: Data of calibration models for Clorazepate, Diazepam and Etizolam drug by QuEChERS extraction and LC-MS/MS analysis.

3.3.2 Precision and Recovery

ANOVA (Analysis of Variance) was performed to evaluate precision. The study resulted in a value termed RSD (Relative Standard Unit). This study compared accuracy and recovery values to those reported previously (Zilfidou et al., 2019). The study's working samples fulfilled accuracy limitations, with no observed deviations exceeding 20%. Recovery values varied from 70% to 120% of the acceptable range. The extraction recovery of each analyte was also evaluated. [27].

Extraction recovery is a measure of how efficiently an analyte is removed from a sample during extraction. This computation is used to evaluate the efficacy of the extraction method utilised in the study. Two sample sets were prepared for analysis, with one requiring extraction of an extractable matrix (AE) and subsequent spiking. Before extraction (BE), the other set was spiked in a blank working matrix. The study's recovery rates were calculated using these sets. The recovery for the BE sample was calculated using the following formula:

Recovery (%) = BE/AE × 100

To test recovery, two sets of samples were used: one was produced in a blank matrix spiked after extraction (AE), and the other was spiked using two equations:

Clorazepate, Diazepam, and Etizolam recovery rates in urine samples were between 74 and 117%. QuEChERS exhibited importance by achieving 70.4-100% recovery in urine samples at the lowest concentration, such as 5 ng/ml. Table 3 shows the analytes obtained concentrations and recovery percentages, which are both within the optimal range of 70-120%.

S.no.	SPIKED CONCENTRATION	URINE	
		Extracted concentration (ng/mL)	Recovery %
Clorazepate			
1	5	3.7	74
2	10	11.05	110.5
3	20	22.37	111.85
4	50	51.57	103.14
5	100	102.11	102.11
6	200	193.61	96.805

S.no.	SPIKED CONCENTRATION	Urine	
		Extracted concentration (ng/mL)	Recovery %
Etizolam			
1	5	3.73	74.6
2	10	10.58	105.8
3	20	23.57	117.85
4	50	50.86	101.72
5	100	103.6	103.6
6	200	192.63	96.315

S.no.	SPIKED CONCENTRATION	Urine	
		Extracted concentration (ng/mL)	Recovery %
Clorazepate			
1	5	5.17	103.4
2	10	9.21	92.1
3	20	18.31	91.55
4	50	45.92	91.84
5	100	96.5	96.5
6	200	208.87	104.435

Table 2. The recovered concentration of CZP, DZP, EZM in Urine

3.3.3. Matrix Effect:

Analytes were tested for matrix effects at two different concentrations: 200 ng/ml for HQC and 5 ng/ml for LQC. The goal of this investigation was to see if the sample matrix affected the precision and accuracy of the analytical data. The study found that all analytes had matrix effects with variations of less than 20% at both LQC and HQC levels. This indicates that the matrix effects did not surpass a 20% divergence from predicted results. This conclusion is acceptable based on the study's parameters and meets required validation requirements.

Analytical technique validation often involves keeping matrix effects within a 20% variance to provide accurate and reliable results. The investigation confirms the validity and robustness of the analytical technique by proving that matrix effects for all analytes were within acceptable ranges. The approach may offer exact readings even in complicated sample matrices.

Relative Matrix Effect= (Peak area of matrix matched standard)/ (Peak are of standard solution), Relative Matrix effect should be less than 20%.

M.E= (A-B/A)*100

4. Conclusion:

The LC-MS/MS technology extracts antianxiety medications from urine at six different concentrations. The approach was verified using SWGTOX criteria, resulting in high precision and accuracy across various concentrations. Endogenous chemicals found in urine samples did not interfere with the results. The extraction

procedure used in the investigation yielded consistent and repeatable drug recoveries, even at low concentrations of 5 ng/ml, demonstrating its dependability. This instrument is essential for investigating drug and toxin dissolution, absorption, and degradation. It helps us understand how they interact with uric acids and enzymes, which is important for toxicology research. It helps develop and improve analytical procedures for identifying and quantifying chemicals in urine, leading to more accurate and reliable forensic examinations.

The developed and validated approach is very sensitive and repeatable for drug analysis. This approach allows for simultaneous detection of numerous substances.

The test is simple and quick, making it ideal for high-throughput analysis. Forensic investigations require speedy processing of large volumes of samples to ensure full analysis. This method improves operating efficiency in forensic laboratories by reducing processing time to 8 minutes. It speeds up the investigative process by allowing forensic professionals to assess materials within a reasonable time frame. Fast processing speeds up forensic testing and research. In general, it improves productivity and output, allowing for more efficient forensic investigations.

The QuEChERS extraction was optimised using low sample and solvent consumption under ideal conditions, using methanol as the solvent, a mixture of sodium acetate and magnesium sulphate salts for the salting-out effect, a vortex or homogeniser for agitation, and PSA with magnesium sulphate for sorbent cleanup. The method's validity and demonstration of desirable linearity, intermediate precision, repeatability, and accuracy. The approach's recovery rate ranged between 75 and 119%. R2, the goodness of fit parameter, the technique's accuracy in relation to repeatability, and relative percentage standard deviation values of less than 20% were all significant. As a result, the approach was effectively applied to examine biological data.

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